# Adenosine Diphosphate Ribosylation of G Proteins by Pertussis and Cholera Toxin in Isolated Membranes. Different Requirements for and Effects of Guanine Nucleotides and $Mg^{2+}$

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**ADP ribosylation of membranes by pertussis toxin (PT) and cholera toxin (CT) was studied as a function of addition of ATP, various guanine nucleotides, $Mg^{2+}$, and inorganic phosphate ($P_i$). ADP ribosylation of a 40 kilodalton (kDa) band by PT is markedly enhanced by ATP and GTP and is strongly inhibited by $P_i$ or $Mg^{2+}$. GTP analogs (GTP$\gamma$S and GMP-adenyl-5′-yl imidodiphosphate) were less effective. In contrast, ADP ribosylation of two substrates for CT (of 42 and 50 kDa) is stimulated by $P_i$, $Mg^{2+}$, and GTP or GTP analogs such as GTP$\gamma$S, but is unaffected by ATP. These stimulatory conditions correlate well with GTP-mediated activation of stimulated nucleotide-binding regulatory component of adenyl cyclase. Optimal conditions for ADP ribosylation by PT do not correlate simply with conditions thought to lead to stabilization of an inactive form of inhibitory nucleotide-binding regulatory component of adenyl cyclase ($G_i$) or $G_i$-like protein; rather, the data suggest the involvement of both a stimulatory nucleotide site on PT (positively effected by either ATP or GTP) and a stabilizing site on the PT substrate (affected by GDP, GDP$\beta$S, or GTP). Treatment of membranes with Lubrol PX increased ADP ribosylation by PT by as much as 25- to 30-fold, but inhibited the action of CT. Using defined conditions for ADP ribosylation by PT and CT, distinct labeling patterns were observed in thyroid, brain, corpus luteum, liver, heart, and erythrocytes membranes. All membranes were more intensely labeled by PT rather than CT. (Molecular Endocrinology 1: 472–481, 1987)**

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## INTRODUCTION

Many hormones regulate cellular responses by coupling to and activating specific guanine nucleotide-binding regulatory components termed G (or N)*1*/proteins (1, 2). These G proteins are susceptible to ADP ribosylation by the bacterial toxins from *Vibrio cholera* [cholera toxin (CT)] and/or *Bordetella pertussis* [pertussis toxin (PT)]; also islet-activating protein. Stimulatory G ($G_s$), which stimulates adenylyl cyclase (3), is substrate for only CT (4) while inhibitory G ($G_i$), which is inhibitory to the enzyme (1), and $G_o$, and some $G_p$, coupled to polyphosphatidylinositide hydrolysis, are ADP ribosylated by PT (5–7). Transducin is a substrate for both toxins (8) while the function of $G_o$ is unknown. $G_s$ (3), transducin (9), $G_o$ (7), and putative $G_i$s (10) have been purified. They have a heterotrimeric structure, of the $\alpha\beta\gamma$-type (11), having interchangeable $\beta$- and $\gamma$-subunits (12), and differ in their $\alpha$-subunits, which are the sites for ADP ribosylation. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), ADP ribosylated $\alpha$-subunits of $G_s$ have apparent molecular masses of 42–45 and 50–52 kilodaltons (kDa), termed here $\alpha_{s1}$ and $\alpha_{s2}$, respectively (13), while the PT substrates are 39–41 kDa (14). The 40–41 kDa bands ADP ribosylated by PT may be $\alpha$-subunits of $G_i$ and $\alpha$-subunits of other G proteins, such as $G_p$. CT and PT affect G protein transduction differently. ADP ribosylation of $G_s$ facilitates its activation by GTP (15), while ADP ribosylation of $G_i$ by PT inhibits its activation by GTP (5, 16), which blocks its coupling to inhibitory receptors (5, 17, 18).

In thyroid gland, the effects of CT (19), guanine nucleotides (20, 21), and stimulation of cAMP formation have been characterized. Inhibitory regulation of cAMP

formation by both norepinephrine and acetylcholine has also been reported in the thyroid, but only that of norepinephrine appears to be sensitive to PT (22, 23). Acetylcholine appears to act in a $G_i$-independent manner (23).

The present work examined conditions for CT and PT ADP ribosylation of thyroidal G proteins. Optimal conditions for ADP ribosylation of $\alpha_s$s by CT toxin are actually inhibitory for the ADP ribosylating action of PT, and vice versa. Lubrol PX markedly increased ADP-ribosylation by PT but inhibited the effect of CT.

## RESULTS

Figure 1 illustrates the effect of adding inorganic phosphate ($P_i$), ATP, guanine nucleotide, and $Mg^{2+}$ alone and in combinations on the incorporation of [$^{32}$P]ADP-ribose from [$^{32}$P]NAD$^+$ into CT and PT substrates of thyroid membranes. In the absence of toxins (Fig. 1A) no labeling was seen in 38,000 to 50–52,000 $M_r$ polypeptides, the range of toxin-mediated substrate ADP ribosylation. Toxin independent labeling of the 56 kDa polypeptide was most marked with $Mg^{2+}$ and $P_i$ and suppressed by 1 mM each of ATP and GTP (lanes 7 and 8 of Fig. 1).

In agreement with previous studies (5, 16), CT and PT promoted ADP ribosylation of different proteins. PT labeled a 40 kDa ($\alpha_i$) and CT labeled two peptides, $\alpha_{s1}$ and $\alpha_{s2}$, of 42 and 50 kDa, respectively. GTP and ATP with absence of $Mg^{2+}$ and $P_i$ provided the best labeling of the pertussis substrate. In the presence of $Mg^{2+}$, GTP was more effective in promoting ADP ribosylation by PT than ATP. The relative availability of the two substrates to CT is similar in thyroid membranes. On the other hand, ADP ribosylation by CT was best observed at high concentrations of $P_i$ with GTP and Mg ion; ATP had no effect on the action of CT.

### Role of Incubation Conditions on Labeling by Toxins

The result of varying the concentrations of the different reagents present during ADP ribosylation reactions was studied. Increasing concentrations of unlabeled NAD$^+$, added to assays containing 0.18 μM [$^{32}$P]NAD$^+$ (0.45 μCi/pmol) plus both 10 μg/ml PT and 100 μg/ml CT, decreased the incorporation of [$^{32}$P]ADP ribose into the PT and CT substrates (Fig. 2). Significant reductions of incorporation of [$^{32}$P]NAD$^+$ were obtained between 10 and 30 μM unlabeled NAD$^+$ for both toxins. On the basis of this, the remaining studies used 10 μM NAD$^+$ (~0.036 μCi/pmol, $4 \times 10^6$ cpm/60 μl assay).

### Effect of $P_i$ and $Mg^2$

$P_i$ inhibits ADP ribosylation by PT and stimulates ADP ribosylation by CT (Fig. 3). Inhibition is obtained at much lower concentrations than stimulation. As indicated in Fig. 1 $Mg^{2+}$ inhibited the effect of PT but did not have a major effect in enhancing ADP ribosylation by CT.

Incorporation of radioactivity into the 40 kDa PT substrate and $\alpha_{s1}$ and $\alpha_{s2}$ substrates for CT is linearly dependent on membrane concentration up to 36 μg/60-μl assay regardless of addition of ATP or GTP (not shown). The effects of GTP and ATP on PT ADP ribosylation were apparent at all concentrations of membranes tested.

### Effects of Nucleotides

Figure 4 illustrates that GTP and ATP were most effective on PT stimulation of incorporation of [$^{32}$P]ADP ribose into the 40 kDa band. GTP analogs were least effective (compare lane 1 to lanes 4 and 6 of Fig. 4) while GDP and GDPβS were of intermediate effectiveness. Labeling with ATP at 0.1 and 1 mM was equivalent, while 1 mM of GTP was maximal (Fig. 5). The effect of 0.01 mM ATP was greater than that of 0.01 mM GTP. ATP increased ADP ribosylation of the 40 kDa band by PT even at saturating GTP (Fig. 5).

Concentrations as low as 0.3 μM free $Mg^{2+}$ inhibited ADP ribosylation by 50–60% in the absence of nucleotides; with ATP, 100 to 300 μM were needed for similar inhibition and somewhat higher concentrations were required when GTP was present (Fig. 6). With both ATP and GTP the potency of $Mg^{2+}$ inhibition was increased 3-fold (not shown). The effect of $Mg^{2+}$ was not due to stimulation of hydrolysis of ATP or GTP by the membranes (not shown).

Since either ATP or GTP enhanced the formation of ADP ribosyl-$G_i$ in the presence of saturating concentrations of the other, we examined the possibility that more than one binding site was involved. Since ATP stimulated ADP ribosylation of tubulin by PT (24), and since the α-subunits of G proteins bind guanine nucleotides (25), we speculated that ATP enhanced PT activity while guanine nucleotides interact with the PT substrate to alter its substrate quality. This hypothesis was tested examining competitive interaction between ATP and/or GTP and GDPβS. GDPβS should block the effect of GTP by competitively occupying its postulated site on the PT substrate but have no effect on the action of ATP, postulated to interact with the toxin. However, the results did not support the hypothesis. GDPβS (100 μM) did not decrease the effect of 10 μM GTP (Fig. 7).

Using purified $G_i$ from human erythrocytes the affinities of GTP and GDPβS were about equal (26). AMP-adenyl-5′-yl imidodiphosphate [AM-P(NH)P] has an effect similar to that of ATP, but requires higher concentrations (Fig. 7B), and its action is also potentiated by GDPβS. The effect of AMP-P(NH)P was not due to contaminating ATP since it was the same after purification by diethylaminoethyl (DEAE)-Sephadex chromatography (27) which removed more than 99% of a trace amount of [α-$^{32}$P]ATP.

GDPβS enhanced ADP ribosylation to some extent and shifted the dose-response curve for ATP towards higher concentrations by blocking the effect of 1, 3, and

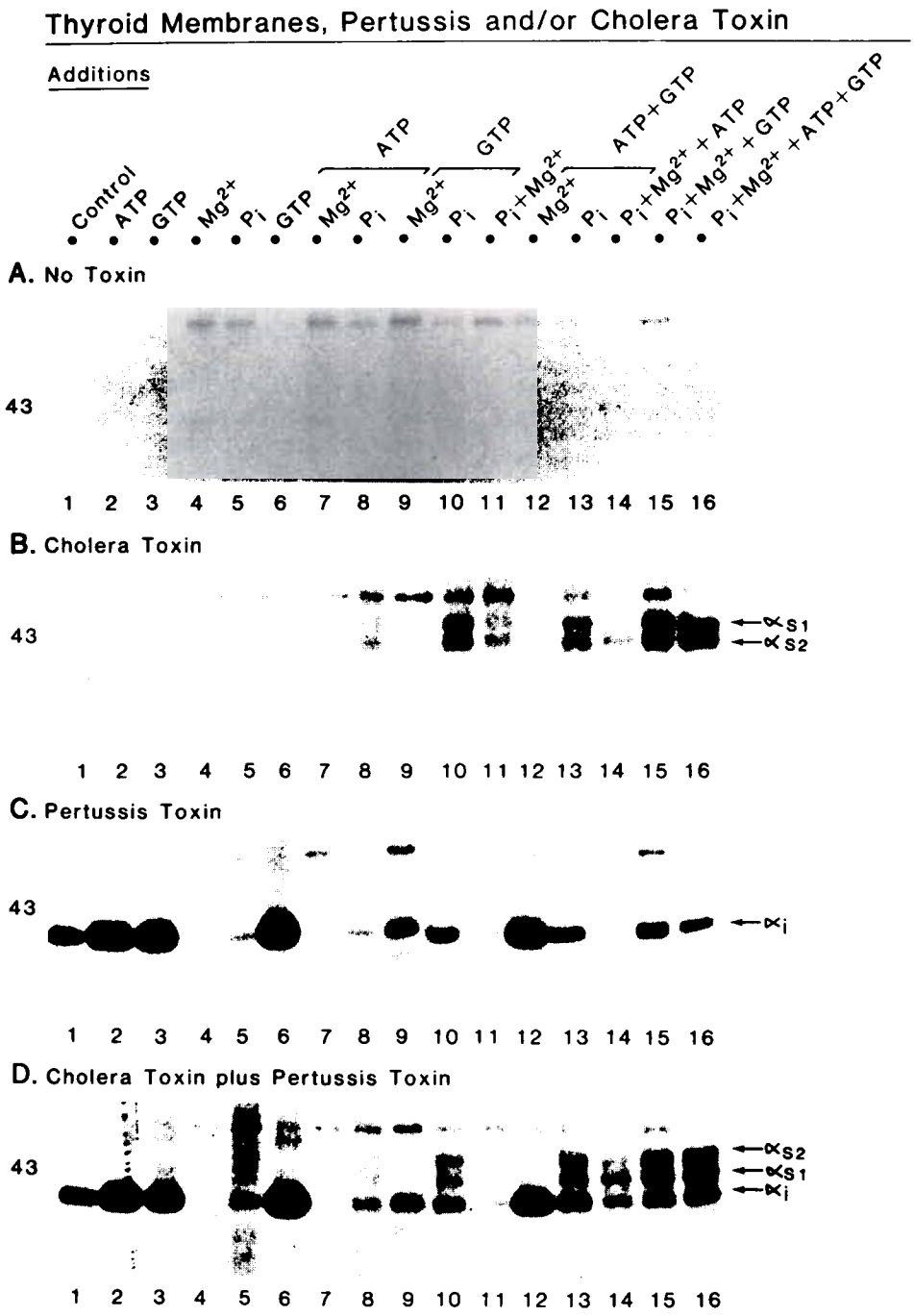


**Fig. 1.** Effect of ATP, GTP, $P_i$; and $Mg^{2+}$ Alone and in Combination on Labeling of Protein Bands in Thyroid Membranes by PT and/or CT

The position of the ovalbumin molecular weight standard run in parallel lanes is indicated by *43*. When present ATP and GTP were 1 mM, $P_i$ was 300 mM, and $MgCl_2$ was 10 mM. In this and the subsequent figures the position of identifiable toxin substrates ($\alpha_{s1}$, $\alpha_{s2}$, and $\alpha_i$) are indicated on the *right margin of autoradiographs*. For further experimental details see *Materials and Methods*.

10 $\mu$M ATP and potentiating higher concentrations of ATP (Fig. 8).

In contrast to GDP$\beta$S, a wide range of GTP analogs, GDP$\gamma$S and GMP-P(NH)P, had no effect on ADP ribosylation obtained at 1 $\mu$M ATP (not shown). GDP$\beta$S caused a right shift in the dose response curves for GTP and GTP$\beta$S when CT stimulated ADP ribosylation of $\alpha_{s1}$ and $\alpha_{s2}$ (Fig. 9). ATP has no effect on Basal (Fig. 1) or GTP-stimulated (not shown) incorporation of ADP ribose into $\alpha_{s1}$ and $\alpha_{s2}$ by GTP.

**Effect of Lubrol PX on ADP Ribosylation by PT**

In parallel studies with pure human erythrocyte $G_i$, we observed that Lubrol PX enhanced ADP ribosylation when it was added to the assay. As illustrated in Fig.

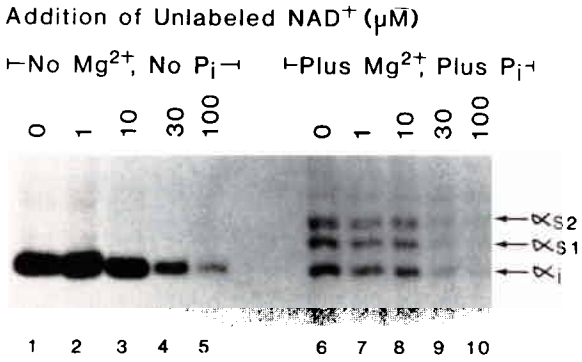


**Fig. 2.** Effect of Increasing Concentrations of Unlabeled $NAD^+$ on Labeling of Toxin Substrates with [$^{32}$P]ADP ribose

The reactions contained both 10 μg/ml PT and 100 μg/ml CT, plus 1 mM ATP and 1 mM GTP, and, when present, 300 mM $P_i$ and 10 mM $MgCl_2$. For details see *Materials and Methods*.

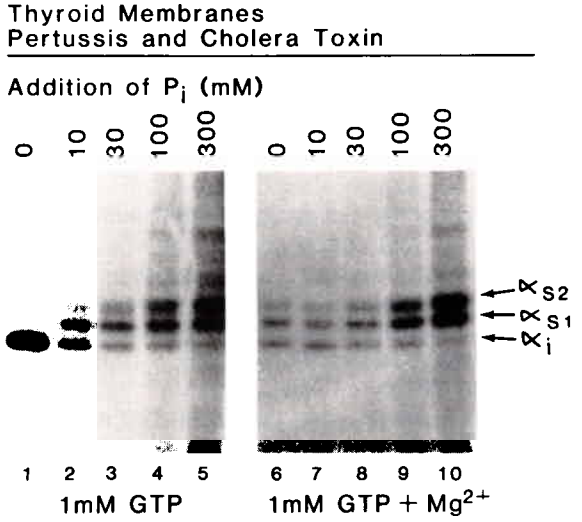


**Fig. 3.** Effect of Increasing Concentrations of $P_i$ on Incorporation of [$^{32}$P]ADP-Ribose into Toxin Substrates as Measured in the Absence of ATP

The reactions contained both 10 μg/ml PT and 100 μg/ml CT, the indicated concentrations of $P_i$ (mM), and 1 mM GTP and, when present, 10 mM $MgCl_2$. For the rest of the conditions see *Materials and Methods*.

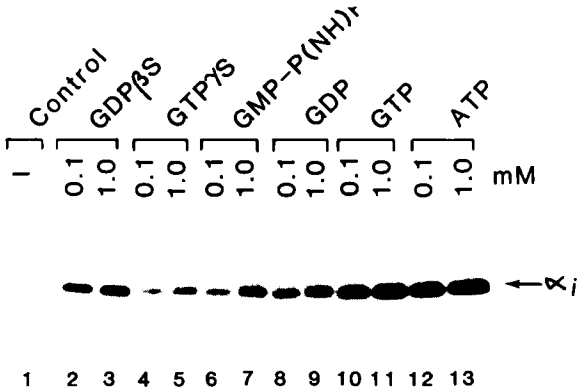


**Fig. 4.** Effect of Various Nucleotides on ADP Ribosylation of PT Substrate

When present, nucleotides were 0.1 and 1.0 mM. For the rest of the conditions see the figure and *Materials and Methods*.

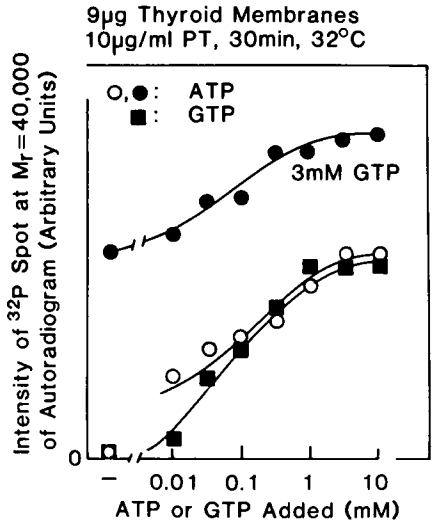


**Fig. 5.** Quantitative Plot of the Intensities of the Autoradiographic Spots as a Function of Nucleotide Concentration

Relative intensities were derived from an autoradiogram with low exposure, so as to avoid film saturation, and represent the integrated areas under the scanned spots. ■, Effect of increasing concentrations of GTP; O, effect of increasing concentrations of ATP; ●, effect of increasing concentrations of ATP in the presence of 3 mM GTP.

10, mixing the membranes with Lubrol PX before their incubation for ADP ribosylation by PT enhanced labeling of the 40 kDa PT substrate. As little as 0.003% Lubrol PX was effective and 0.3% was maximal resulting in a 25- to 30-fold increase in ADP ribosylation of the 40 kDa substrate. Lubrol PX did not interfere with any of the effects of nucleotides, $Mg^{2+}$ or $P_i$ described above (not shown). Lubrol PX had either no effect (0.003–0.1%) or inhibited (0.1–1%) labeling of the $\alpha_s$ substrates by CT (not shown).

### Labeling of Membranes from Other Tissues

Using conditions that are optimal for ADP ribosylation with CT and PT, in the thyroid, we examined ADP ribosylation of membranes from rat corpora lutea, rat brain, dog heart, rat liver, and human erythrocytes. While all these tissues have substrates for both toxins, qualitative and quantitative differences in both the size of the $\alpha_{s2}$ bands and the relative quantities of $\alpha_{s1}$ to $\alpha_{s2}$

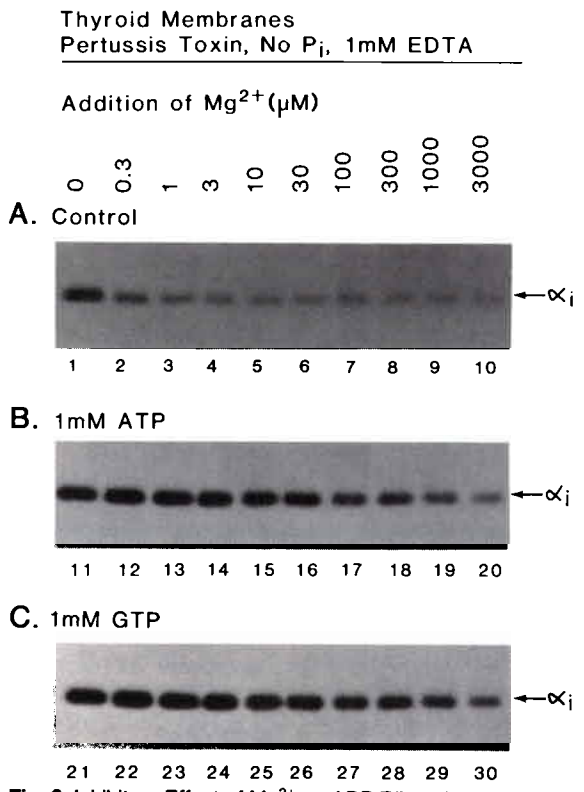


**Fig. 6.** Inhibitory Effect of $Mg^{2+}$ on ADP Ribosylation by PT as Seen in the Absence (A) or the Presence of ATP (B) or GTP (C)

The reactions contained varying concentrations of $MgCl_2$ to give the final concentrations of $Mg^{2+}$, and when present, 1 mM ATP or 1 mM GTP. Exposure of the X-ray film in A was 2.5 times longer than that used to obtain the autoradiograms of B and C. For the rest of the conditions see the figure and *Materials and Methods*.

exist (Fig. 11). In addition, large differences in the relative abundance of the CT and PT substrates are apparent in different tissues.

## DISCUSSION

The present results demonstrate that the conditions that are optimal for ADP ribosylation by CT and PT in the thyroid are quite different. It is possible that the conditions which have been used could influence the turnover of the toxin substrates. The conditions that are optimal for CT action suggest a functional correlation with $G_s$ activity states based on adenylyl cyclase assays. This is not true for the effect of PT on the activity state of $G_i$. The results with PT are compatible with more than one nucleotide binding site involving, possibly, both the substrate and the toxin. This situation probably explains why conditions used in the literature for ADP ribosylation with CT are rather uniform, while those used for ADP ribosylation with PT vary markedly. Thus, CT treatment which activates $G_s$ with respect to adenylyl cyclase regulation is commonly carried out in the presence of GTP (or analogs) and $Mg^{2+}$. The stim-

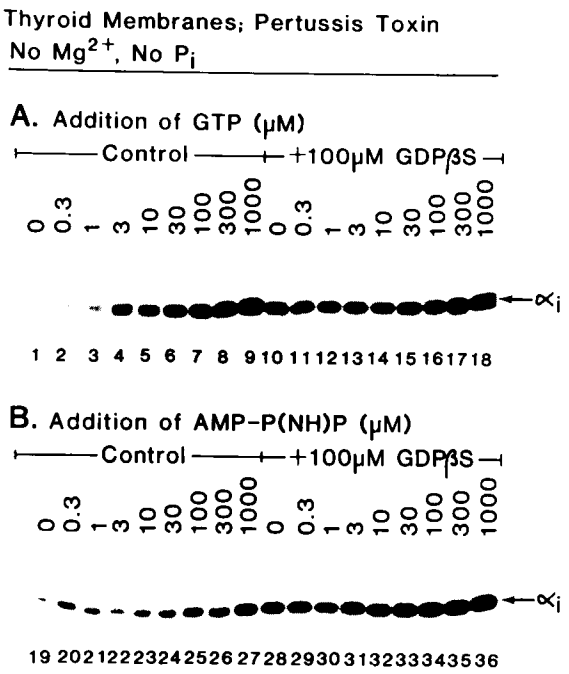


**Fig. 7.** Stimulation of PT-Mediated ADP Ribosylation

A, Lack of effect of GDPβS on the stimulation of GTP of PT-mediated ADP ribosylation. Concentrations of GTP added varied between 0 and 1000 μM. B, Effect of GDPβS on the stimulation by AMP-P(NH)P of PT-mediated ADP ribosylation. The reactions contained no ATP or GTP, the indicated concentrations of AMP-P(NH)P, and, when present, 100 μM GDPβS.

ulatory effect of high concentrations of $P_i$ was noted by Kaslow *et al.* (28) and is probably an effect on the specific activity of the toxin, for it is also seen with ADP ribosylation of arginine (29). On the other hand, ADP ribosylations with PT have been done with (5, 30) or without (31) ATP, with GTP (5, 30, 31), or GMP-P(NH)P (32) with (5, 30, 31) or without (32) $Mg^{2+}$, or, with the GDP analog, GDPβS (30). Only a fraction of the PT substrate present was ADP ribosylated since the studies were done with $P_i$.

The conditions optimal for ADP ribosylation with CT are consistent with activation of $G_s$ in the assay of adenylyl cyclase. Thus, addition of GTP or GTP analogs, but not ATP, stimulated incorporation of ADP ribose, while GDPβS completely inhibited the stimulatory effects of GTP analogs. Moreover, $Mg^{2+}$ plus GTP, which promotes activation of $G_s$, further enhanced ADP ribosylation. Kahn and Gilman (33), working with purified $G_s$, found similar conditions for its activation and ADP ribosylation with CT. However, studies with the purified protein showed an absolute requirement for an additional membrane component (ADP-ribosylation factor) without which ADP ribosylation did not occur (34) and which was previously noted as a stimulatory factor in cytosol (35).

The results obtained with PT in intact membranes strongly suggest involvement of two nucleotide sites with different specificities, each being important in determining the incorporation of ADP ribose $Mg^{2+}$ was more or less inhibitory depending on the nucleotide

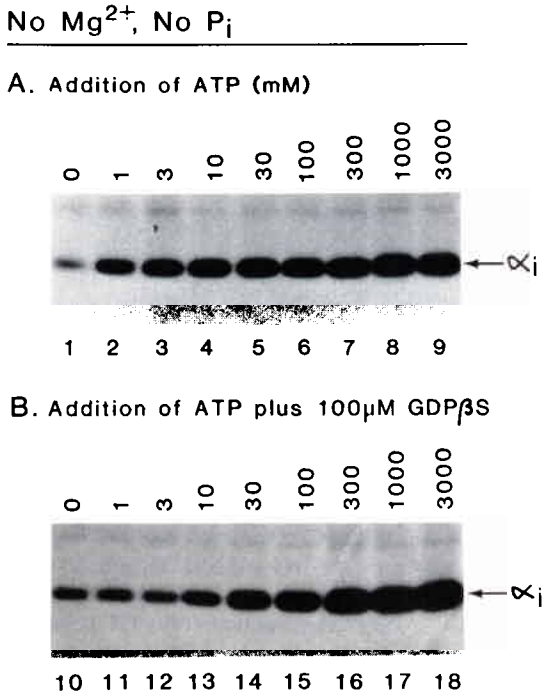


**Fig. 8.** Inhibitory and Stimulatory Effects of GDPβS on the Stimulation by ATP of PT-Mediated ADP Ribosylation

The reactions contained no GTP, the indicated concentrations of ATP in the absence (A) and presence (B) of 100 μM GDPβS. For the rest of the conditions see the figure and *Materials and Methods*.

added. The guanine nucleotide-stimulated ADP ribosylation was less sensitive to inhibition by $Mg^{2+}$ than the ATP-stimulated reaction. Although the mechanism involved in the ATP and guanine nucleotide sites are undefined, experiments with PT and pure human erythrocyte $G_i$ in solution (36) indicated two interactions: 1) ATP with the toxin, possibly by facilitating the dissocia-

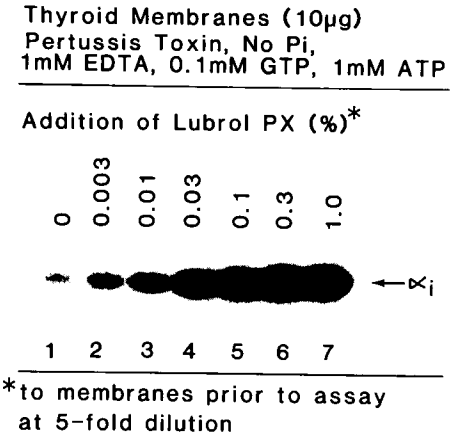


**Fig. 10.** Effect of the Nonionic Detergent Lubrol PX on ADP Ribosylation by PT

Membranes were exposed to the indicated concentrations of Lubrol PX at 4 C for 30 min and then added to the ADP ribosylation assay mixture with Lubrol PX at 4 C for 30 min, and then added to the ADP ribosylation assay mixture with preactivated toxin, incubated for 30 min, and analyzed as described in *Materials and Methods*. Autoradiography was for 2 h.

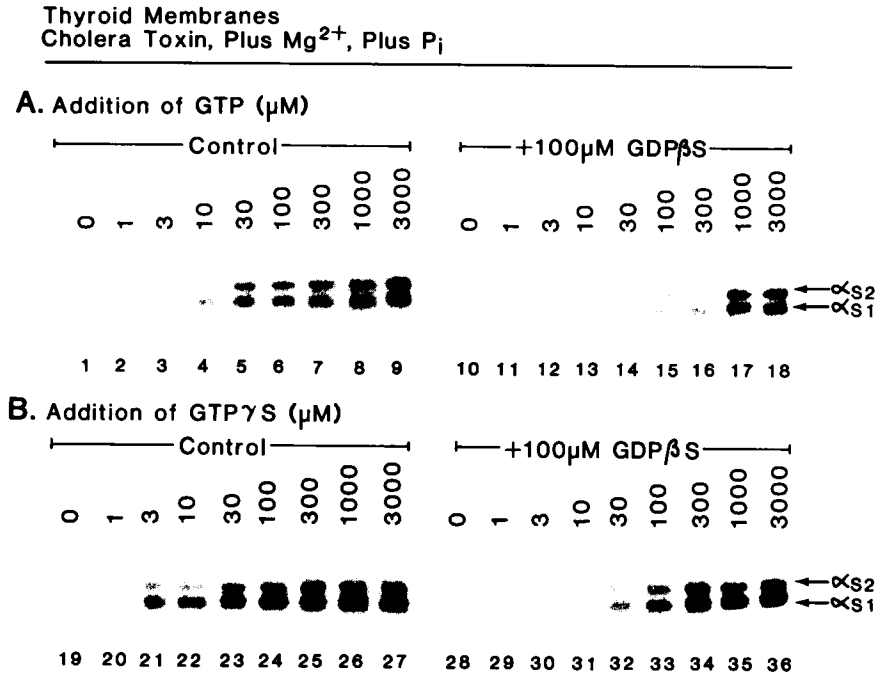


**Fig. 9.** Competitive Inhibition by GDPβS of GTP- and GTPγS-Stimulated ADP Ribosylation of $\alpha_{s1}$ and $\alpha_{s2}$ by CT

The reactions contained 10 mM $MgCl_2$, 300 mM $P_i$, the indicated concentrations of GTP (A) or GTPγS (B), and when present, 100 μM GDPβS. ATP was absent. For the rest of the conditions see the figure and *Materials and Methods*.

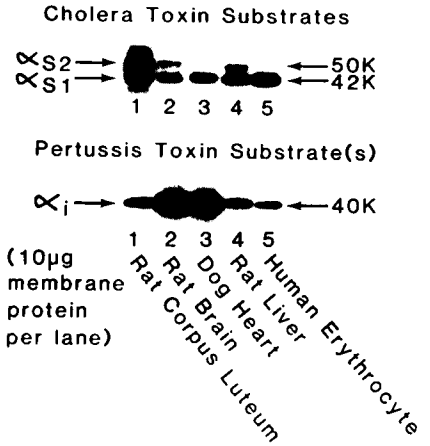


**Fig. 11.** ADP Ribosylation of CT and PT Substrates in Corpus Luteum, Brain, Heart, Liver, and Erythrocyte Membranes

The composition of the incubation medium was described under *Materials and Methods* using 10 μg membrane protein, either 100 μg/ml CT (*upper panel*), or 10 μg/ml PT (*lower panel*), and 1 μM $NAD^+$ ($10 \times 10^6$ cpm/assay). The reactions with CT contained in addition: 1 mM GTP, 10 mM $MgCl_2$, and 300 mM $P_i$, pH 7.5. The reactions with PT contained in addition: 1 mM ATP and 1 mM GTP, but no $MgCl_2$ and no $P_i$. Incubations were for 60 min to ensure that the reactions had gone to completion. *Top panel*; Photograph of the autoradiograph obtained after 21 h of exposure at −70 C. *Bottom panel*; Photograph of the autoradiograph obtained after 4 h of exposure at −70 C.

tion of the $\alpha_{s1}$ subunit from the rest of the molecule (37); and 2) GTP and its analogs with both the toxin (where they mimick the effect of ATP) and the substrate, which they stabilize to heat inactivation, especially in the absence of $Mg^{2+}$. Thus the additivity of ATP and GTP represents both of these interactions. GDP mimicks both effects of GTP, albeit with a much slower time course (36), which explains the lack of competitive interaction between GTP and GDP. Although GDPβS also interacts with both the toxin and its substrate, it does not activate the toxin and does not stimulate ADP ribosylation by PT. Very likely, the reduction in ADP ribosylation caused by $Mg^{2+}$ reflects interaction of this ion with the PT (36).

The results with Lubrol PX suggest that the PT substrate in the membranes is not totally accessible for ADP ribosylation. Since the detergent also enhances labeling of pure $G_i$ (albeit to a smaller extent of at most 2- to 3-fold), it is not clear how much of the effect reflects a conformational change of the substrate and/or toxin and how much is due to disruption of vesicular structures making the substrate physically more accessible to the toxin.

The ADP ribosylation results demonstrate via toxin-mediated ADP-ribosylation the dual presence of $G_s$ and $G_i$ in the thyroid. The thyroid $G_s$ demonstrates size heterogeneity in its α-subunit, with $\alpha_{s1}$ and $\alpha_{s2}$ having a molecular mass of 42 and 50 kDa, respectively, on SDS-PAGE. The abundance of ADP-ribosyl-$\alpha_{s1}$ was essentially the same as that of ADP-ribosyl-$\alpha_{s2}$. In contrast, only one labeled band, migrating at approximately 40 kDa, was obtained with PT. Since thyroid adenylyl cyclase is under inhibitory hormonal control, a true $G_i$ is part of the 40 kDa band but existence of one or more additional substrates of the same molecular mass as $G_i$ cannot be excluded.

A comparison of labeling patterns obtained with thyroid membranes to those obtained with other tissues (fig. 11), reveals striking differences. Two CT substrates were found in some (thyroid, liver, brain, and corpus luteum), but not in others (red blood cells and heart). When two CT substrates were present their relative abundance varied from tissue to tissue. The ratio was greater than unity in the corpus luteum, unity in the thyroid and less than unity in brain and liver. The apparent size of $\alpha_{s2}$ in brain appears to be slightly larger than in liver. No significant differences in the mobilities of the $\alpha_{s1}$ bands were found among the different tissues, suggesting the 42 kDa bands reported here are equivalent to the 45 kDa bands observed by others (3). Recent work from our laboratory (38), as well as that of Gilman and co-workers (39), has shown that the two forms of $\alpha_s$ are encoded in two separate mRNA species indicating that they are not artifacts introduced during preparation of the membranes. The functional significance of these differences between the CT substrates is not known at this time.

In all the tissues examined there appeared to be only a single substrate of about 40 kDa for PT. This may be an oversimplification since purification of guanine nucleotide binding proteins from brain membranes revealed two PT substrates, termed $\alpha_i$ and $\alpha_o$ (7). They differ in mass by only about 1000–2000 daltons. More recently, PT substrates in fat (40) have been separated into a doublet. Thus, the PT-labeled band may represent more than one substrate, especially in brain, thyroid, and heart, where labeling was found to be most intensive. On the other hand, purification of $G_i$ from human erythrocytes revealed only a single $\alpha_i$ in these cells (10, 14).

In all the tissue studies the ratio of the PT-labeled bands was greater than the CT-labeled bands. The ratio of 4–5 in human erythrocytes is in good agreement with direct protein purification results (10, 14). Similarly purification of PT substrates and $G_s$ from brain, revealed at least a 100-fold excess of PT substrates over $G_s$.[1] The larger proportion of PT substrate with respect to CT substrate in brain than in erythrocytes is also evident from Fig. 11. The PT substrates in the thyroid may be in a similar range of abundance as brain. The differences in membrane CT and PT substrates documented here via ADP ribosylation and observed on purification of the substrate proteins almost certainly contributes to the differences in the type of transduction mechanisms observed in these membranes.

## MATERIALS AND METHODS

### Materials

Carrier-free $^{32}P_i$ in 0.1 N HCl was purchased from CitiChem (Tuxedo, NY). AMP-P(NH)P, 3′-AMP, GTP, GTPγS, GMP-

[1] Codina, J., and L. Birnbaumer, unpublished.

P(NH)P, GDP, GDP$\beta$S, $NAD^+$, nicotinamide mononucleotide, glyceraldehyde-3-phosphatediethylacetal, glyceraldehyde-3-phosphate dehydrogenase (~100 U/mg), phosphoglycerate kinase (~450 U/mg), lactate dehydrogenase (rabbit muscle, ~550 U/mg), and NAD-pyrophosphorylase (~0.15 U/mg) were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). ATP, prepared by phosphorylation of adenosine, creatine phosphate, thymidine, spermine, dithiothreitol, Tris, EDTA (free acid), HEPES, BSA (Cohn fraction V), DNAse 1, myokinase (rabbit muscle, 1000–1500 U/mg), and creatine phosphokinase (rabbit muscle, 100–150 U/mg) were purchased from Sigma (St. Louis, MO). DEAE-Sephadex A-25, $T_4$ polynucleotide kinase, and P-1 nuclease were from Pharmacia-PL Biochemicals (Piscataway, NJ). The reagents used for SDS-PAGE and Dowex resins were from Bio-Rad (Rockville Centre, NY). Precoated plastic 20 × 20-cm sheets of polyetheleneimine-cellulose for TLC of nucleotides (Machery-Nagel Polygram CEL PEI) were purchased from Brinkmann Instruments (Westbury, NJ). PT was purified from *Bordetella p.* cultures (41) and CT was purchased from Sigma Chemical Co. or List Laboratories (Campbell, CA). Solutions of chemicals and biochemicals were made with glass distilled $H_2O$ and, when appropriate, neutralized to pH 7.0–7.5 with Tris-base or HCl.

### Methods

SDS-PAGE was according to Laemmli (42) in 10% polyacrylamide gel slabs (ratio, acrylamide to bis-acrylamide, 100:2.6) of 15 cm × 18 cm × 1.5 mm. Samples (20–30 $\mu$l) were solubilized in Laemmli's sample buffer (42) with 1% SDS and Pyronin Y as tracking dye. After electrophoresis at 100–150 V, the gel slabs were stained with 0.2% Coomassie blue in acetic acid-methanol-water (5:5:1), destained in 10% acetic acid, dried under vacuum between cellophane sheets, and subjected to autoradiography at −70 C between 12 and 72 h using Kodak XR-5 film and two DuPont Lighting Plus BE intensifying screens.

Ascending TLC was developed with either 1 M LiCl, 0.8 M ammonium sulfate (pH 5.7), or 2 M sodium formate, pH 4.0. These solvent systems allowed the separate identification of ATP, GTP, ADP, GDP, AMP, GMP, $P_i$, $NAD^+$, NMN, and ADP ribose and were used to follow the progress of the reactions leading to the synthesis of [$\gamma$-$^{32}$P]ATP, [$\alpha$-$^{32}$P]ATP, and [$\gamma$-$^{32}$P]GTP from $^{32}P_i$, and of [$^{32}$P]$NAD^+$ from [$\alpha$-$^{32}$P]ATP as well as the determination of hydrolysis of nucleotides by membranes.

[$\alpha$-$^{32}$P]ATP, [$\gamma$-$^{32}$P]ATP and [$\gamma$-$^{32}$P]GTP (43) and [$^{32}$P]NAD (44) were synthesized in the Molecular Endocrinology Core Laboratory of the Baylor College of Medicine Diabetes and Endocrinology Research Center.

### Purification of AMP-P(NH)P

Commercial AMP-P(NH)P (10 $\mu$mol in 100 $\mu$l, pH 7.5, adjusted with Tris-base, containing $10^6$ cpm 153Ci/mmol [$^{32}$P]ATP) was purified by DEAE-Sephadex chromatography using a linear gradient from 0–600 mM $(NH_4)_2HCO_3$ (27). The elution of [$\alpha^{32}$P]ATP and AMP-P(NH)P was followed by determination of $^{32}P$ and absorbance at 260 nm, respectively. They were separated by better than 99%. The peak fractions of AMP-P(NH)P were pooled, lyophilized, resuspended in distilled $H_2O$ (yield 60%), and kept frozen at −20 C.

### Preparation of Membranes

Bovine thyroid membranes were prepared at 0–4 C by a modification of the procedure of Suzuki *et al.* (45). Briefly, well minced fresh thyroid tissue was suspended in 10 vol 1 mM EDTA and 25 mM Tris-HCl, pH 7.5, homogenized with 15 strokes in a Dounce homogenizer with a loosely fitting pestle and filtered through a no. 12 Japanese silk screen. After centrifugation at 800 × *g* for 10 min, the pellet was discarded and the supernatant was centrifuged at 20,000 × *g* for 20 min. The supernatant was carefully aspirated and discarded. Crude thyroid membranes were obtained by floating off the upper two-thirds less colored portion of the pellet with homogenization medium. These membranes were then washed three times with homogenization medium and resuspended in small aliquots in the same medium at a protein concentration of 2.5–5.0 mg/ml. Adenylyl cyclase activity which is stimulated between 4- to 5-fold by TSH in a GTP-dependent manner, remained stable for up to 3 months at −70 C.

Rat liver membranes were prepared by an abbreviated (46) method of Neville (47). Rat corpus luteum membranes were prepared (48) from superovulated rat ovaries (49) on the seventh day after human CG administration. Rat brain membranes (P2 synaptosomes) were prepared according to Diaz-Arrastia *et al.* (50). Dog heart membranes (sarcolemma) were prepared as described (51). Human erythrocyte membranes were prepared (10) from hypotonic lysates. All membrane fractions were stored in small aliquots at −70 C until used.

### PT and CT

The ammonium sulfate suspension of PT was dialyzed against 50 mM NaCl, 1 mM EDTA, and 25 mM NaHepes, pH 8.0, adjusted to a concentration of 600 $\mu$g/ml, subdivided into 10-$\mu$l aliquots and stored at −70 C. CT (6 mg/ml) was dissolved in $H_2O$, subdivided into 10-$\mu$l aliquots and stored at −70 C. CT (3 mg/ml) and PT (300 mg/ml) were preactivated at 32 C for 30 min with 25 mM DTT. Before use, the preactivated toxins were diluted 5-fold with 25 mM Tris-HCL, pH 7.5, 1.25 mM EDTA, and 0.125–2.5 mg/ml BSA.

### [$^{32}$P]ADP Ribosylation Reactions and Preparation of Samples for SDS-PAGE

Unless stated otherwise, incubations were for 30 min at 32 C in a final volume of 60 $\mu$l containing 10–12 $\mu$l thyroid membranes (*ca.* 10 $\mu$g) and DNAse I (0.1 mg/ml), 20 $\mu$l preactivated and diluted PT (10 $\mu$g/ml) and/or CT (100 $\mu$g/ml) and 1–2 $\mu$g BSA/assay, 10 $\mu$M [$^{32}$P]$NAD^+$ (4–5 × $10^6$ cpm/assay), 10 mM thymidine, 1 mM EDTA, 20 mM Tris-HCl, and, when present: 1 mM ATP, 1 mM GTP, 10 mM $MgCl_2$, and 300 mM potassium phosphate ($P_i$). The final pH of the reactions was 7.5. Reactions were started by addition of [$^{32}$P]$NAD^+$ and stopped with 1.0 ml 20% ice-cold trichloroacetic acid. After standing on ice for 10 min, the mixture was centrifuged in a table top centrifuge at 4 C. The supernatant fluid was aspirated and the pellet was extracted with 1.5 ml ice-cold ethyl ether. After centrifugation in the cold, the supernatant was aspirated, and the tubes were brought to room temperature to evaporate any remaining ether. The membrane pellets were dissolved at room temperature in 20–30 $\mu$l Laemmli's sample buffer (42) containing 1% SDS and pyronin Y. When Lubrol PX was added to the assays, the reactions were stopped by first adding 40 $\mu$l 375 mM NaCl followed by 900 $\mu$l ice-cold acetone. After standing on ice for 10 min, the mixtures were centrifuged in the cold at 2500 rpm for 30 min and the supernatants were discarded. After addition of 1.0 ml 20% ice-cold trichloroacetic acid, the samples were treated as described above in the absence of Lubrol PX.

When free $Mg^{2+}$ was varied, the total $MgCl_2$ concentration required to give the desired concentration of $Mg^{2+}$ was calculated according to the equation:

$$MgCl_2 = Mg^{2+}[1 + A_t/(K_A + Mg^{2+}) + B_t/(K_B + Mg^{2+}) + C_t/(K_C + Mg^{2+})]$$

where $A_t$, $B_t$, and $C_t$ are the total concentrations of EDTA, ATP, and GTP in the assays and $K_A$, $K_B$, and $K_C$ are the equilibrium dissociation constants for MgEDTA, MgATP, and MgGTP at the assay pH of 7.5: $K_A$ = 0.4 $\mu$M and $K_B = K_C$ = 60 $\mu$M. For further details see ref. 52.

**Reproducibility of Results**

All experiments were performed at least twice, with most of them having been done three and more times. SDS-PAGE autoradiographies were done in duplicate with representative results of these replications presented.

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